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Detection of Human Papilloma Virus DNA in Lymph Nodes Extirpated at Radical Surgery for Cervical Cancer Is not Predictive of Recurrence

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In women with recurrent cervical cancer after radical surgery, lymph node metastasis is detectable histologically at the time of surgery in only about 50% of cases. The present study was designed to determine whether the detection of human papilloma virus (HPV) DNA in lymph nodes extirpated at operation, as an indication of micrometastasis, is predictive of recurrence. Using polymerase chain reaction (PCR), a total of 140 lymph nodes from 31 patients with HPV 16 DNA positive primary cervical tumours were tested for the presence of an HPV 16 LCR/E6 gene fragment. HPV 16 DNA was detected in extirpated lymph nodes in 75% (6/8) of patients with recurrence (and who died within 5 years after surgery) and in 70% (16/23) of recurrence-free patients. In only four of the patients with recurrence (three of whom had HPV 16 DNA positive lymph nodes) was metastasis detectable histologically at surgery. HPV DNA positive lymph nodes were found in 91% (10/11) of patients with histologically detectable metastasis at surgery and in 60% (12/20) of patients without metastasis. It is concluded that the presence of HPV DNA in extirpated lymph nodes at cervical cancer operation does not appear to be predictive of tumour recurrence. ***J. Med. Virol. 54:183–185, 1998.***

KEY WORDS: PCR; retrospective analysis; micrometastasis

INTRODUCTION

The involvement of human papilloma virus (HPV) in the development of cervical cancer is unequivocal [zur Hausen, 1996]. Thus, HPV infection, with its ability to transform and immortalise infected cells [Watts et al., 1984; Woodworth et al., 1989], is a prerequisite of the oncogenesis, although cofactors are needed for malignant transformation [zur Hausen, 1989], and the exact mechanism whereby HPV promotes tumourigenesis is not fully understood.

Monoclonal status is a hallmark of neoplasia. It has a strong correlation with certain high-risk virus types, such as HPV 16, 18, 31, 33, 35, 45, 56, and 65. In lesions associated with other HPV types, polyclonal cellular status is the rule [Park et al., 1996]. Metastases from HPV-associated tumours always manifest the same HPV type as the primary tumour, and when integration of the viral DNA is seen in the primary tumour, the same integration pattern is found in the metastasis [Fuchs et al., 1989]. Therefore, detection of HPV DNA in histologically negative lymph nodes has been interpreted as an indication of micrometastasis [Lancaster et al., 1986; Mvula et al., 1994]. However, DNA analysis of lymph node preparations is associated with a risk of false positive results—for instance, if the preparation analysed contains HPV DNA derived from degraded tumour cells, carried to the node by cells of the immune system, or if the lymph node preparation has become HPV DNA contaminated at extirpation. By instead analysing the lymph nodes for HPV mRNA, indicative of active HPV infection in live cells, the risk of false positive conclusion might be eliminated [Czeglédy et al., 1994, 1995; Rose et al., 1994]. However, formalin-fixed tissue is not always suitable for retrospective studies of RNA. Accordingly, despite the risk of overdiagnosis, the present study was designed to assess the presence of HPV DNA in lymph nodes and histological evidence of micrometastasis as predictors of cervical cancer recurrence.

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TABLE I. Recurrence in Relation to Histologically Verified Lymph Node Metastasis and HPV DNA Detected in Extirpated Lymph Nodes From Patients Operated for HPV 16 Positive Cervical Cancer

Group of patients	Lymph node metastasis at surgery	Recurrence	No. of patients	HPV 16 DNA in lymph nodes	
				Positive	Negative
I	+	+	4	3	1
II	+	—	7	7	0
III	—	+	4	3	1
IV	—	—	16	9	7
Total			31	22	9

PATIENTS AND METHODS

Patients

During the period 1985–1991, 85 women with cervical cancer underwent radical hysterectomy and pelvic-paraaortic lymphadenectomy (Wertheim-Meigs method) at the Department of Obstetrics and Gynaecology, Lund University. Using an HPV type-specific polymerase chain reaction (PCR) test, formalin-fixed, paraffin-embedded tumour tissue from these patients was retrospectively analysed for the presence of HPV 16 DNA. Forty-seven percent (40/85) of the preparations were found to be HPV positive. Material was available for study from 31 of the HPV 16 positive patients, 30 with squamous cell carcinomas and 1 with a poorly differentiated adenocarcinoma, ranging in clinical stage from I A2 (n = 27) to II A (n = 2) and II B (n = 2). A total of 157 lymph node preparations (three to nine per patient) from the respective patients were available for retrospective HPV 16 DNA analysis.

The presence of lymph node metastasis at operation, tumour recurrence, and patient death within 5 years after surgery were recorded.

DNA Preparation

To avoid the risk of DNA contamination between samples, two to three small cuts of the paraffin-embedded tissue were taken from each specimen with a disposable scalpel instead of using a microtome. After extraction of the paraffin with 400 μL xylene for 15 min at 37°C, followed by washing with 95% ethanol, the dry tissue was digested with 1.5 mg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) in 400 μL lysis buffer (10 mM Tris-HCl, 5 mM EDTA [pH 7.8], and 4% SDS) at 37°C overnight. Saturated NH_4Ac (150 μL) was added, and the tube was vortexed for 30 sec and then centrifuged at 17,600 × *g* at 4°C for 15 min. The supernatant was transferred to a new tube, and the DNA was precipitated with 900 μL of 100% ethanol for 30 min at −20°C. After centrifugation at 17,600 × *g* for 7.5 min, the resulting pellet was washed once with 500 μL of 95% ethanol. The pellet was dried briefly and then dissolved in 50 μL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.8).

DNA PCR

To investigate the integrity of the DNA extracted from the archival tissues and to confirm the absence of inhibitors, all samples were subjected to PCR amplification of part of the human β-globin gene using a primer pair originally described by Saiki and coworkers [1985].

For the HPV 16-specific PCR, we designed a primer set spanning from the end of the long control region (LCR) to the beginning of the E6 gene, yielding a 93 bp amplimer:

16/31+: CGTAACCGAAATCGGTTGAA

and

16/123 –: TCCTGTGGGTGGTGAAAC

A PCR reaction volume of 25 μL was chosen, containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% w/v Triton X-100, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2% w/v BSA, 0.75 μM of each primer, and 0.625 U Taq polymerase (Promega, Madison, WI) plus 2.5 μL of the specimen. After an initial template thermal denaturation at 93°C for 4 min 30 sec, the following cycle profile was used: 1 min 30 sec at 50°C (annealing), 1 min 30 sec at 72°C (extension), and 1 min 30 sec at 94°C (denaturation). At the end of the 35th cycle, the extension step was prolonged by 5 min. Positive controls (containing 1–10 pg of cloned HPV 16 DNA), negative controls (DNA extract from human lung fibroblasts), and reagent controls were amplified along with the patient specimens. Due precautions were taken to minimise the risk of contaminations by other samples or amplified DNA.

To demonstrate PCR amplimer of expected size, a 5 μL aliquot of the reaction mixture was electrophoresed on a 4% agarose gel (3% NuSieve and 1% SeaKem, FMC Bioproducts, Rockland, ME), the gel being stained with ethidium bromide prior to photography in UV light.

RESULTS

The results of the lymph node HPV DNA analysis and follow-up of the patients are summarised in Table I. Eight of the 31 women with HPV 16 positive primary tumours had tumour recurrence and died within 5 years after the radical surgery (groups I and III). Eleven patients had lymph node metastasis detected at operation (groups I and II). Four (36%) of them belonged to the tumour recurrence group. Of the 20 pa-

tients without histological signs of lymph node metastasis at operation (groups III and IV), 4 (20%) had recurrence within 5 years (group III).

Of the 157 available lymph node specimens from the 31 patients, 140 were positive by the β-globin PCR and could therefore be evaluated for the presence of HPV 16 DNA. HPV 16 DNA was detectable in one or more lymph node preparations in 71% (22/31) of cases: in 91% (10/11) of cases where lymph node metastasis was detected histologically at operation, and in 60% (12/20) of cases without histological signs of metastasis at operation, representing 75% (6/8) of the recurrence subgroup and 70% (16/23) of the recurrence-free subgroup.

DISCUSSION

The present findings suggest that neither the histological status of extirpated lymph nodes nor HPV 16 DNA analysis is of use in predicting recurrence. Lymph node metastasis was detectable histologically at operation in only 50% (4/8) of cases characterised by recurrence (lung and/or peritoneal metastasis) and death within 5 years after surgery, and in 30% (7/23) of cases characterised by 5-year recurrence-free survival. The prevalence of HPV 16 DNA lymph node positivity was similarly high both in the recurrence and recurrence-free subgroups (75% [6/8] vs. 70% [16/23]), results which are consistent with findings by a Danish group, though they reported somewhat lower prevalences [Hording et al., 1995].

Moreover, it can be questioned to which extent a finding of HPV DNA positivity in lymph node material constitutes evidence of the existence of live tumour cells capable of inducing metastasis. Owing to the extremely high sensitivity of the PCR technique, which is capable of detecting viral DNA in the presence of only a few molecules, there is a risk of false positive results being obtained from lymph node analysis. This is due to contamination with free viral DNA or single cells from the primary tumour (by instruments, gloves, blood, etc.) of lymph node material extirpated at operation. Another possible source of false positive results is the presence of dead or damaged tumour cells or cellular debris trapped in the lymph nodes. These two sources of misleading results can be eliminated if, instead of testing for the presence of viral DNA, we test for viral expression in the form of mRNA of the E6/E7 genes. Using this approach, only HPV infection in live cells is monitored. In a previous study, this technique was used in a prospective analysis of snap-frozen node tissue. Consistent with the above hypothesis, fewer lymph nodes were HPV 16 E6/E7 mRNA positive than were HPV 16 DNA positive [Czeglédy et al., 1995]. However, results from clinical follow-up of these patients are still not available. It should also be borne in mind that successful extirpation of lymph nodes with micrometastasis should reduce the risk of tumour recurrence, and that, in the present series, had the lymph nodes been left unattended, most of the patients with HPV DNA positive lymph nodes might have had disease relapse.

In the present retrospective study, the sensitivity and specificity of HPV DNA analysis in predicting recurrence would have been as low as 75 and 30%, respectively. Thus, HPV DNA analysis of extirpated lymph nodes at surgery for cervical cancer does not appear to be a viable method of identifying women at risk of tumour recurrence.

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